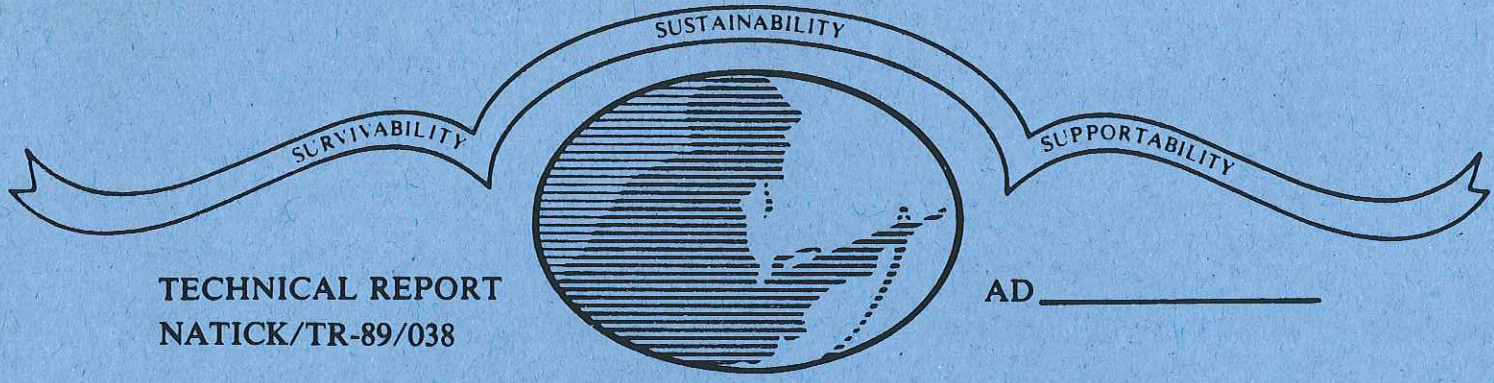

TECHNICAL REPORT
NATICK/TR-89/038

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THE PRODUCTION, PURIFICATION AND PROPERTIES OF THE BIOPOLYMER LEVAN PRODUCED BY THE BACTERIUM *ERWINIA HERBICOLA*

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The optimized conditions for the production of levan by Erwinia herbicola were determined to be pH (6.0-7.5), incubation time (72 hours), culture media (K_2HPO_4, 3.0g; NaCl, 2.0g; Urea, 0.3g; $MgSO_4 \cdot 7H_2O$, 0.4g; $FeSO_4 \cdot 7H_2O$, 0.10g, 5% sucrose per liter), and polymer purification (sodium dodecyl sulfate treatment, dilution with H_2O, dialysis, and methanol precipitation). While continuous culture fermentation at pH 7.2 produced the highest yield of polymer, molecular weight determinations of the isolated polymer could not be made with certainty because of levan behavior in aqueous solution. In addition, the yields of polymer were low (below 25%) when compared to other biopolymers studied (i.e., pullulan, elsinan). The physical properties of clear films produced from this polymer could not be adequately evaluated due to brittleness, which could not be overcome with the addition of glycerol as a plasticizer.

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INTRODUCTION

With the intent of correlating culture methodology, processing conditions, physical properties, and molecular weight (MW), the authors began attempts to produce and characterize the biopolymer levan and evaluate its unique properties for potential material applications, such as low oxygen permeable biodegradable films for food packaging and fibers.

As part of the biopolymer project, researchers examined levan for ease of purification and processing, maximization of yield, and control of MW with large-scale production in mind. In addition, films formed from the purified polymer were evaluated.

Levan is an extracellular B-fructan biopolymer built of anhydro-D-fructofuranoside units with 2:6' glycosidic linkages and an average of 10-12 monomer units between branch points.[1,2] It is produced by several bacteria, including Erwinia herbicola, Streptococcus salivarius, Pseudomonas prunicola, Bacillus subtilis, and Actinomycetes sp., when grown in sucrose media.[1] Bacterial levan is macromolecular[16] and arborescent[17] as opposed to plant levan, which is composed of short unbranched chains.[24]

PREFACE

This report contains the results of a study performed to determine fermentation and processing conditions for the production of the biopolymer levan produced by the bacterium Erwinia herbicola, characterization of the physical properties of the polymer, and determination of the physical properties of films formed from the purified polymer. This study was funded under the U.S. Army Natick Research, Development and Engineering Center (Natick) Program Element 61101A on Biopolymer Production for Varied Military Applications, Project No. 1L161101A91A, Task No. 14, Work Unit AH5202039. The work was undertaken from April 1987 to February 1989.

We thank Dr. Derek Ball and Dr. Elwyn Reese (this laboratory) and Dr. James Rollins (Worcester Polytechnic Institute, Worcester, MA) for their technical support.

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INTRODUCTION

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Past research on culture and harvest conditions for levan production has had several foci. The effects of carbon source and length of incubation on levan production have been examined by Ueda, et al.[3] for the bacterium Aeromonas hydrophila, while Bodie, et al. examined the effects of pH and time on the production of levan by Arthrobacter sp.[4] The effects of time, pH, temperature, sucrose concentration, and inoculum percent on a wide range of levan-producing bacteria were examined by Mattoon, et al. in an effort to screen for the best levan-producing system on a cost versus yield basis.[5] Culture and levan harvest conditions have also been explored, on a limited basis, for the bacteria Streptococcus salivarius and Streptococcus bovis[18], and the fungus Actinomyces viscosus.[19]

Some general determinations of MWs of levan have been accomplished with viscometry,[5] while Marshall and Weigel estimated MW by gel permeation chromatography[6], Bahary and Stivala used light scattering techniques.[20] Except for the general observation that longer incubation resulted in a more highly branched and higher MW levan,[21] no correlation of culture methodology and MW has been made. It was the purpose of this study to develop this correlation for the bacterium Erwinia herbicola.

MATERIALS AND METHODS

Cultures

Erwinia herbicola strain ATCC 15552 was chosen for this study after comparison with ATCC 11142 Acetobacter pasteurianus, ATCC 15953 Microbacterium

laevaniformans, and QMB 1624 Bacillus coagulans (B. subtilus-pumilus) for recoverable yield of levan on Avigad medium.[8] Cultures were maintained on nutrient agar slants.

Media

The following media were used, in grams per liter of distilled H_2O:

(1) Kato and Shiosaka (K&S)[7] - K_2HPO_4, 2.0; NaCl, 2.0; $MgSO_4 \cdot 7H_2O$, 0.4; $FeSO_4 \cdot 7H_2O$, 0.01; peptone, 2.0 (Difco, Detroit, MI); pH 5.4

(2) Avigad[8] - NH_4Cl, 2.2; NaCl, 5.0; $NaHPO_4 \cdot 2H_2O$, 3.5; $NaH_2PO_4 \cdot H_2C$, 0.8; yeast extract, 3.0 (Difco); pH 7.2

(3) Levan Medium - K_2HPO_4, 3.0; NaCl, 2.0; Urea, 0.3; $MgSO_4 \cdot 7H_2$), 0.4' $FeSO_4 \cdot 7H_2$, 0.01

(Magnesium and Iron solutions were prepared as 100x stocks, filter sterilized, and added aseptically after autoclaving.)

Initial studies were performed using 10% sucrose (commercial grade), but later changed to 5% because of improved yield.[9] The sucrose solution was also prepared separately and added aseptically after autoclaving. Preliminary studies were conducted with the K&S medium, varying the carbon, phosphate and nitrogen source in order to arrive at the best combination of media components. The result of this effort is reflected in the Levar Medium described above.

Culture Conditions

For the continuous culture a BioFlow Model C30 Fermenter (New Brunswick Scientific, New Brunswick, NJ) with a 1.8L culture vessel was set with an agitation rate of 200 rpm, at temperature of 25°C, and aeration of 0.5 L/min. Flow rates were controlled with a Rabbit Peristaltic Pump (Rainin Instrument Co., Inc., Woburn, MA) for 8 days. Cells were removed with a model RC-5 Sorvall Refrigerated Centrifuge (DuPont Instruments, Wilmington, DE) equipped with a GSA rotor spun at 23,430 X g for 30 minutes.

To inoculate cultures, researchers scraped growth, using a sterile inoculating loop, from the nutrient agar slants on which the strains were maintained and transferred to a 250 mL DeLong flask containing 50 mL of media. This culture was grown for 24 hours under batch conditions, and a new flask inoculated with 2% inoculum from the growing culture. The second flask was grown for 24 hours and used as the inoculum for the test cultures. Batch conditions involved shaking at 25°C to 30°C and 125 or 160 rpm in an Environ-shaker (Lab-Line Instruments, Inc., Melrose Park, IL).

Processing and Purification

Several methods of purification were tried in an attempt to separate the cells from the polymer and obtain a pure product. Initially (method A), the pH of the supernatant was adjusted to 7.0 with 1 N NaOH and diluted 1:2 with distilled water. Then a 1:2 volume of acetone was added while stirring, and the water-acetone solution was decanted off. The precipitate was washed

several times with acetone and dried over $CaSO_4$ in a desiccator. A variation on this procedure (method B) in which the initial pH adjustment was replaced with the addition of 1% (v/v) 10% n-alkyldimethylbenzyl ammonium chlorides was tried, as was a further variation (method C), in which methanol was used as a precipitating agent.

The method finally adopted (method D), involved the addition of 1% (w/v) sodium dodecyl sulfate (SDS) to the DeLong flask, which was then shaken at 125 rpm for 4 hours. Milli-Q water (Millipore Corp., Bedford, MA), twice the volume of the culture, was added and the mixture was spun as before. SDS and other low molecular weight contaminants were removed by dialysis against Milli-Q water using Spectrapor 12,000 MW cutoff membrane tubing (Thomas Scientific, Swedesboro, NJ). The final solution was precipitated with twice the volume of methanol and centrifuged at 650 g for 15 minutes. The methanol/water was decanted off and the precipitate washed with methanol and dried over $CaSO_4$ in a desiccator under vacuum.

Purification of the polymer from the continuous culture system was achieved by centrifugation of the fermentation medium and subsequent use of a Tangential Flow Filtration Unit with Pellicon Model Cassette System OM-141 (Millipore Corp., Bedford, MA) on the supernatant. The levan solution was passed through a 0.45 m cassette to remove particulate impurities, then the filtrate was passed through a cassette with a 30,000 MW cutoff to selectively retain the high MW polymer (>30,000) while allowing the lower MW impurities to pass through. The concentrated retentate was then precipitated with acetone as in purification method A described earlier.

Analytical Methods

Levan MW distribution and dispersity was determined on a Waters 150-C ALC/GPC Gel Permeation Chromatograph (Waters Chromatography Div., Millipore Corp., Milford, MA) using 3 Biogel TSK columns according to the procedures described in Wiley, et al.[10] Standards and samples were solubilized in the carrier solvent; 0.1% in an aqueous solution of 0.1M sodium acetate; 2% acetic acid (v/v) and 0.05% sodium azide (w/v). Injection volume was 200-300 L and run time was 40 minutes.

Light-scattering techniques[14,15] were also attempted as an additional determinant of the MW of the levan produced during continuous culture. Low-Angle Laser Light Scattering (LALLS) assays were performed using a Multiple Angle Laser Light Scattering Photometer, Dawn Model F (Wyatt Technology, Santa Barbara, CA). A solution of 10^{-5} g/mL levan was dissolved in running buffer consisting of 2% acetic acid and 0.1M sodium acetate, pH 4.0.

The composition of levan was characterized using a High Performance Liquid Chromatograph (Waters Chromatography Div., Millipore Corp., Milford, MA) equipped with a Model 6000 solvent delivery system; differential refractometer detector, sensitivity 1 X 10^{-7} refractive index units; Model U6K universal injector; and a Carbohydrate Analysis, 30 cm X 3.9 mm I.D., stainless steel column (Waters). Chromatography was performed at ambient temperature (ca. 25°C) using acetonitrile:water mixtures (75:25 or 80:20) with a flow-rate of 2 mL min^{-1}, sample injection 5 L. The system was calibrated using fructose (Pfanstiehl), glucose (Eastman), and sucrose (Fischer) standards, and standard

curves were determined. For quantification, peak height was measured to determine concentration. The samples were hydrolyzed for one hour with 10% formic acid prior to injection.

The levan was characterized by Nuclear Magnetic Resonance (NMR) analysis and compared with a levan standard and an inulin standard provided by Dr. Elwin Reese of this laboratory and a sample of levan from a different bacterium provided by the USDA.[23] A Varian XL 200 Superconducting FT NMR Spectrometer with a 47 kG Nb-Ti magnet was used for analysis. ^{13}C spectra were measured for 6-10% w/v solutions in D_2O (10mm sample tubes) at 50.3 MHz. External TMS was the reference (=0) for assigning chemical shift values.

Film Casting

Films of the purified levan were formed in bioassay trays 243 mm x 243 mm 18 mm (Cole Palmer, Chicago, IL). Two solutions were used to cast films, a solution of Milli-Q water and biopolymer at 54 g per mm^2, and the same solution with up to 10% glycerol added as a plasticizer. The solutions were degassed and poured into the trays and allowed to evaporate in an oven at 40 to 45°C until dry. The films were then removed from the trays for subsequent analysis of mechanical properties.

RESULTS

Batch Culture

Erwinia herbicola was the only strain to produce harvestable polymer among those selected for screening. Since prior research[22] had determined that the polymer produced by this bacterium was typical bacterial levan, it was chosen for further study. Table 1 shows the effects of incubation time on "gross yield" of levan. The term gross yield is used because during solubilization of levan for Gel Permeation Chromatographic analysis, microscopic examination revealed cell debris in many of the samples. After changing processing conditions, the amount of cell debris seen during microscopic examination was greatly reduced, and the yield from these samples is referred to as "actual yield". Tables 2 and 3 show the effect of length of incubation on actual yield at a given pH. The effect of incubation time and pH on yield seems to indicate a peak in yield around the third day, followed by a slow decline. This is very close to the 48-60 hour window found by Avigad.[1]

The selection of 25°C as the optimum temperature for levan production was based on observations made by Matoon, et al.[5] that this lower than normal temperature (for bacterial growth) increased levan yield in the strain 248 of an unidentified polysaccharide producing organism they screened for levan production.

The effects of carbon, phosphate, and nitrogen sources on gross yield are shown in Tables 4, 5 and 6, respectively, using the Kato and Shiosaka

TABLE 1

EFFECT OF INCUBATION PERIOD ON LEVAN YIELD

USING ATCC 15552 ERWINIA HERBICOLA[a]

Sample No.[b]	Incubation (days)	Initial pH	Gross Yield g	Gross Yield %[c]
L-6	2	7.2	0.952	9.5
L-3	3	7.2	1.841	18.4
L-4	4	7.2	0.977	9.8
L-5	5	7.2	3.6	d
L-7	6	7.2	4.5	d
L-8	7	7.2	3.5	d

[a]Conditions: Avigad Medium; 10% sucrose; 30°C; Environ-shaker 125 rpm; 50 mL/250mL DeLong flask; 4% inoculum; processing method (A).

[b]N=1

[c]Yield based on conversion of sucrose to levan.

[d]Not processed further. Brown gummy material.

TABLE 2

EFFECT OF INCUBATION PERIOD ON LEVAN YIELD AT pH 6.0 AND 7.0 USING ATCC 15552 ERWINIA HERBICOLA[a]

Sample No.[b]	Incubation (hours)	Initial pH	Mean Actual Yield g	Mean Actual Yield %[c]
1	18	6.0	0	0
2	24	6.0	0	0
3	36	6.0	0.431	0.86
4	48	6.0	0.078	1.56
5	60	6.0	0.087	1.72
6	72	6.0	0.342	6.84
7	84	6.0	0.304	6.08
8	96	6.0	0.170	1.26
9	18	7.0	0.031	0.63
10	24	7.0	0.029	0.57
11	36	7.0	0.123	2.45
12	48	7.0	0.135	2.69
13	60	7.0	0.171	3.41
14	72	7.0	0.211	4.22
15	84	7.0	0.142	2.84
16	96	7.0	0.126	2.51

[a]Conditions:Levan Medium; 5% sucrose; 27°C; Environ-shaker 125 rpm; 50 mL/250mL DeLong flask; 2% inoculum; processing method (D).

[b]N=2

[c]Yield based on conversion of sucrose to levan.

TABLE 3

EFFECT OF INCUBATION PERIOD ON LEVAN YIELD AT pH 5.0 AND 8.0 USING ATCC 15552 ERWINIA HERBICOLA[a]

Sample No.[b]	Incubation (hours)	Initial pH	Mean Actual Yield g	Mean Actual Yield %[c]
17	18	5.0	0	0
18	24	5.0	0	0
19	36	5.0	0	0
20	48	5.0	0	0
21	60	5.0	0.057	1.13
22	72	5.0	0.086	1.72
23	84	5.0	0	0
24	96	5.0	0	0
25	18	8.0	0	0
26	24	8.0	0	0
27	36	8.0	0	0
28	48	8.0	0	0
29	60	8.0	0	0
30	72	8.0	0.152	2.99
31	84	8.0	0	0
32	96	8.0	0	0

[a]Conditions: Levan Medium; 5% sucrose; 27°C; Environ-shaker 125 rpm; 50 mL/250 mL DeLong flask; 2% inoculum; processing method (D).

[b]N=2

[c]Yield based on conversion of sucrose to levan.

medium (1). Sucrose resulted in the best yield at 7.1%, with raffinose following at 4.9% (Table 4). Among the phosphate sources (Table 5), there were several with similar gross yields; likewise, there was no clearly preferred nitrogen source (Table 6). In these cases, nutrient source was selected on the basis of both yield and color of polymer, with preference for a medium resulting in a clean (cell-free), white polymer.

The effect of pH on yield was a bit more complex. In the initial experiment, which examined only gross yield, pH had little effect on yield (Table 7). A wider range of pH values was studied and differences in actual yield were then seen (Table 8), with the extreme pH values of 5 and 8, resulting in little to no product.

Continuous Culture

Table 9 shows the results of producing levan by continuous fermentation. Samples were taken at specified intervals (Table 9) and after termination of the continuous run the contents of the fermenter were also processed. The yields of levan were higher than most other methods evaluated and the product was relatively clean and free from cell debris (actual yield = gross yield). The MW distribution of the product was characterized by high dispersity (4.0-9.0) during the initial period up to 72 hours and a lower dispersity (1.4-2) thereafter.

TABLE 4

EFFECT OF CARBON SOURCE ON LEVAN YIELD

USING ATCC 15552 ERWINIA HERBICOLA[a]

Sample No.[b]	Carbon Source (10%)	Initial pH	Gross Yield g	Gross Yield %[c]
L-20	Sucrose	6.8	0.705	7.1
L-21	Dextrose	6.8	0.033	0.3
L-22	Galactose	6.8	0.129	1.3
L-23	Fructose	6.8	0.015	0.2
L-24	Maltose	6.8	0.080	0.8
L-25	Mannose	6.8	0.82	0.8
L-26	Raffinose	6.8	0.4787	4.9
L-27	Mannitol	6.8	0.136	1.4

[a]Conditions: K & S Medium; 25°C; Environ-shaker 160 rpm; 3 days incubation; 50 mL/250 mL DeLong flask; 2% inoculum; processing method (A).

[b]N=1

[c]Yield based on conversion of sucrose to levan.

TABLE 5

EFFECT OF PHOSPHATE SOURCE ON LEVAN YIELD

USING ATCC 15552 ERWINIA HERBICOLA[a]

Sample No.[b]	Phosphate Source	%	Initial pH	Gross Yield g	Gross Yield %[c]
L-45	KH_2PO_4	0.10	6.8	2.44	24.4
L-44	KH_2PO_4	0.20	6.8	2.71	27.1
L-49	K_2PO_4	0.10	6.8	2.46	24.6
L-50	K_2PO_4	0.30	6.8	2.35	23.5
L-46	Na_2HPO_4	0.35	6.8	1.43	14.3
	NaH_2PO_4	0.08			
L-47	Na_2HPO_4	0.40	6.8	1.98	19.8
L-51	Na_2HPO_4	0.70	6.8	2.09	20.9
L-48	NaH_2PO_4	0.40	6.8	1.18	11.8

[a]Conditions: K & S Medium; 10% sucrose; 25°C; 160 rpms Environ-shaker; 50 mL/250 DeLong flask; 3 days incubation; 2% inoculum; processing method (A).

[b]N=1

[c]Yield based on conversion of sucrose to levan.

TABLE 6

EFFECT OF NITROGEN SOURCE ON LEVAN YIELD

USING ATCC 15552 ERWINIA HERBICOLA[a]

Sample No.[b]	Nitrogen Source	%	Initial pH	Gross Yield g	Yield %[c]
L-28	$NaNO_3$	0.20	6.8	0.090	0.9
L-29	Yeast Ex.	0.02			
	Peptone	0.20	6.8	0.092	0.9
L-30	Yeast Ex.	0.20			
	Peptone	0.20	6.8	0.208	2.1
L-31	$NaNO_3$	0.20			
	Peptone	0.20	6.8	0.104	1.0
L-32	Urea	0.03			
	$(NH_4)_2S)_4$	0.01	6.8	0.183	1.8
L-33	Yeast Ex.	0.10			
	Peptone	0.10	6.8	0.074	0.7
L-34	Urea	0.03	6.8	0.303	3.0
L-35	Yeast Ex.	0.10			
	Peptone	0.20	6.8	0.074	0.7
L-36	Peptone	0.10			
	$NaNO_3$	0.10	6.8	0.010	0.1
L-37	Peptone	0.20			
	$(NH_4)_2SO_4$	0.01	6.8	0	0
L-38	Peptone	0.10			
	Yeast Ex.	0.20	6.8	0.363	3.6
L-39	NH_4Cl	0.22			
	Yeast Ex.	0.30	6.8	0.119	1.2
L-40	NH_4Cl	0.22			
	Yeast Ex.	0.20	6.8	0.125	1.3
L-41	NH_4Cl	0.22			
	Peptone	0.20	6.8	0.154	1.5
L-42	NH_4Cl	0.22			
	Peptone	0.10	6.8	0.061	0.6
L-43	NH_4Cl	0.22			
	Yeast Ex.	0.10			
	Peptone	0.10	6.8	0.123	1.2

[a]Conditions: K & S Medium; 10% sucrose; 25°C; 3 days incubation; Environ-shaker 160 rpm; 50 mL/250 DeLong flask; 2% inoculum; processing method (A).

[b]N=1

[c]Yield based on conversion of sucrose to levan.

TABLE 7

EFFECT OF pH ON GROSS LEVAN YIELD

USING ATCC 15552 ERWINIA HERBICOLA[a]

Sample No.[b]	Initial pH	Gross Yield g	Gross Yield %[c]
L-55	6.0	1.72	17.2
L-56	6.5	1.46	14.6
L-57	6.8	1.76	17.6
L-58	7.0	1.62	16.2
L-59	7.2	1.77	17.7
L-60	7.5	1.32	13.2

[a]Conditions: Levan Medium; 10% sucrose; 25°C; Environ-shaker 160 rpm; 3 days incubation, 50 mL/250 mL DeLong flask; 2% inoculum; processing method (A).

[b]N=1

[c]Yield based on conversion of sucrose to levan.

TABLE 8

EFFECT OF pH ON LEVAN YIELD

USING ATCC 15552 ERWINIA HERBICOLA[a]

Sample No.[b]	Initial pH	Incubation (days)	Mean Actual Yield g	% [c]
18	5.0	1	0	0
2	6.0	1	0	0
10	7.0	1	0.029	0.57
26	8.0	1	0	0
20	5.0	2	0	0
4	6.0	2	0.078	1.56
12	7.0	2	0.135	2.69
28	8.0	2	0	0
22	5.0	3	0.086	1.72
6	6.0	3	0.342	6.84
14	7.0	3	0.211	4.22
30	8.0	3	0.150	2.99
24	5.0	4	0	0
8	6.0	4	0.170	1.26
16	7.0	4	0.126	2.51
32	8.0	4	0	0

[a]Conditions: Levan Medium; 5% sucrose, 25°C; Environ-shaker 125 rpm; 50 mL/250 mL DeLong flask; 2% inoculum; processing method (D).

[b]N=2

[c]Yield based on conversion of sucrose to Levan.

TABLE 9

PRODUCTION OF LEVAN BY CONTINUOUS FERMENTATION

USING ATCC 15552 ERWINIA HERBICOLA[a]

Sample No.[b]	Time (h)	Initial pH	Final pH	Super-natant (mL)	Flow-rate (mL/h)	Yield g	Yield %[c]	Mol. Disp. Wt(K)	Mol. Disp.
L-65	48	7.2	4.25	1470	40.4	30.2	20.5	1617	8.6
L-67	68	7.2	4.76	1645	24.1	49.3	29.9	1124	4.0
L-66	72	7.2	4.18	1360	18.9	26.1	19.2	1507	9.0
L-69	120	7.2	4.37	1660	13.8	31.8	18.9	1359	1.5
L-68	168	7.2	4.67	4425	26.3	130.5	29.5	1348	1.4
L-70	190	7.2	4.17	1350	Ferm.	29.4	21.7	1286	2.0

[a]Conditions: Levan Medium; 10% sucrose; aeration 0.5 L/min; Agitation 200 rpm; 25°C; flow-rates variable; 2% inoculum; tangetial flow purification. Culture incubated 48 hours before starting media flow.

[b]N=1

[c]Yield based on conversion of sucrose to levan.

Extraction and Processing

Method A (adopted from Avigad[11]) in Figure 1 was used initially, but the resulting polymer was often brown and gummy. Further research indicated methanol as an improved precipitating agent,[12] and microscopic evidence of cell debris in the polymer led to the trail of Methods B and C (Figure 1). Method D (Figure 2) was the method of choice and resulted in a clean (microscopically cell-free) white polymer.

Purification

Tangential Flow filtration was used to purify the large volumes (>10 L) of culture media produced from the continuous culture system. This technique could not be used for the small volumes of culture media produced in the batch culture experiments. Vacuum filtration could not be used in the purification process because of the viscosity of the levan solution; even when the volume was doubled with Milli-Q water to reduce viscosity the solution was still too viscous.

Analysis

Gel permeation chromatographic analysis of the levan samples purified from batch and continuous culture showed little or no peak. Increasing the amount of sample injected also failed to produce a response in the analysis. Figure 3 shows the peaks for the levan purified by tangential flow filtration and a levan processed method D. To validate the composition of the isolated polymer,

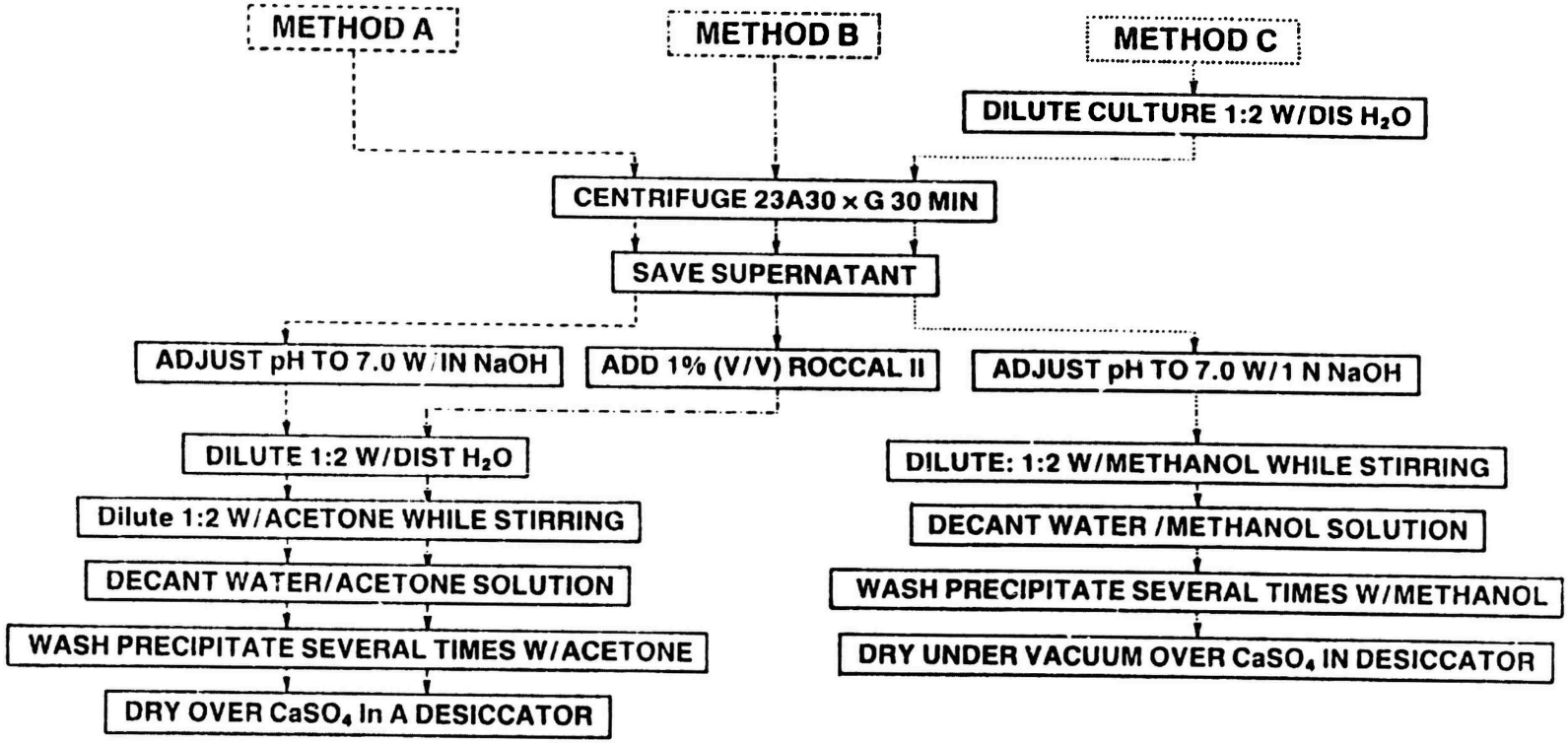


Figure 1. Methods A, B, and C for processing the biopolymer levan.

Add 1% (w/v) SDS to culture, shake 4 hrs 125 rpm

!

!

Dilute 1:2 w/Milli-Q H_2O

!

!

Centrifuge 23,430 x g, 30 min

!

!

Dialyze super. (12.000 MW cutoff) against Milli-Q H_2O

!

!

Dilute 1:2 w/methanol while stirring

!

!

Centrifuge at 650 x g 15 min

!

!

Decant off water/methanol solution

!

!

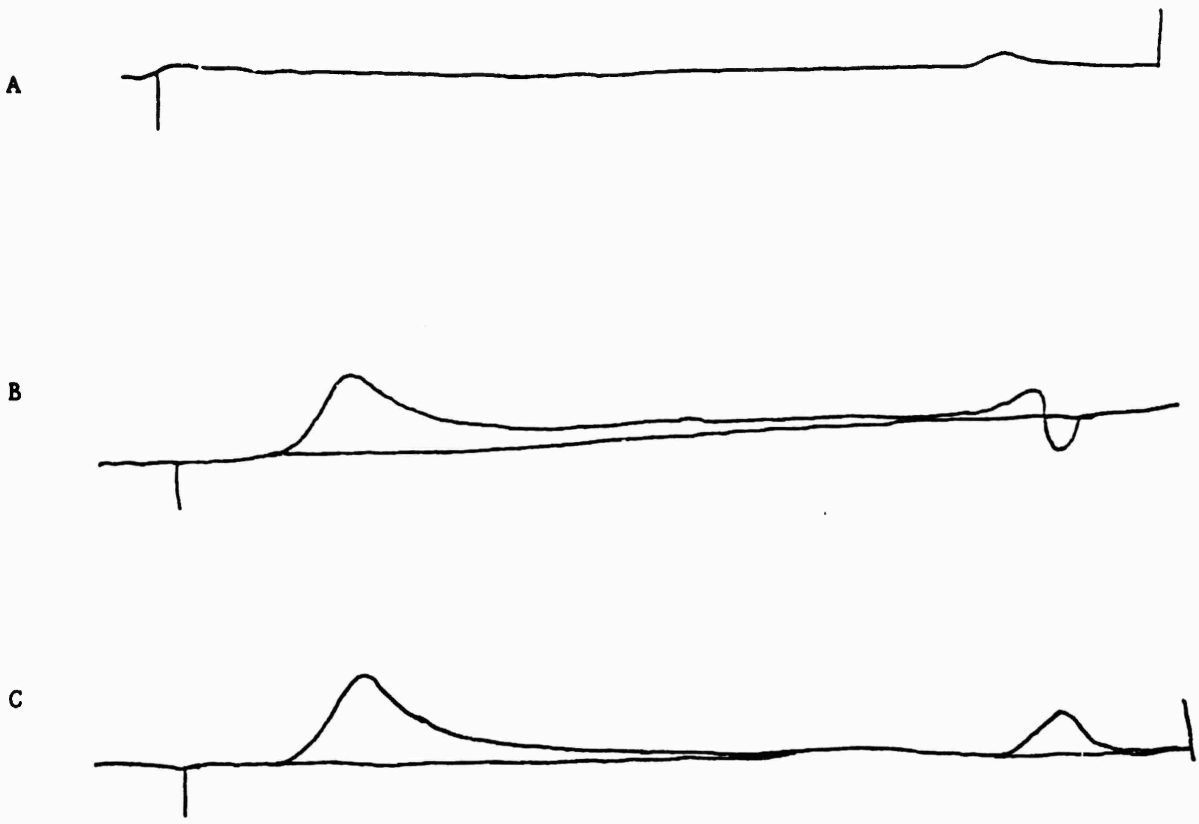
Wash precipitate/pellet with methanol

!

!

Dry under vacuum over $CaSO_4$ in a desiccator

Figure 2. Method D for processing the biopolymer levan.



A Sample 3- Levan processed by method D

B Sample L-70- Levan processed by Tangential Flow

C Sample L-68- Levan processed by Tangential Flow

Figure 3. GPC analysis of levan produced using ATCC 15552 Erwinia herbicola.

liquid chromatography was used to analyze the levan samples. This compositional analysis confirmed that the only sugars present were fructosans (Figure 4). NMR analysis gave further confirmation that the polymer was levan (Figure 5).

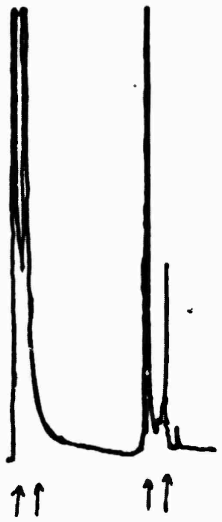
The LALLS technique gave a MW of 125 million for levan from the continuous fermentation, with a particle size of 259 nm and a second viral coefficient of 1.7×10^{-4}.

Film Casting

Solution-cast films made from the resulting polymer were transparent and very brittle. The films shattered upon removal from the tray, which made mechanical testing impossible. This shattering occurred even with films cast with 10% glycerol added as a plasticizer.

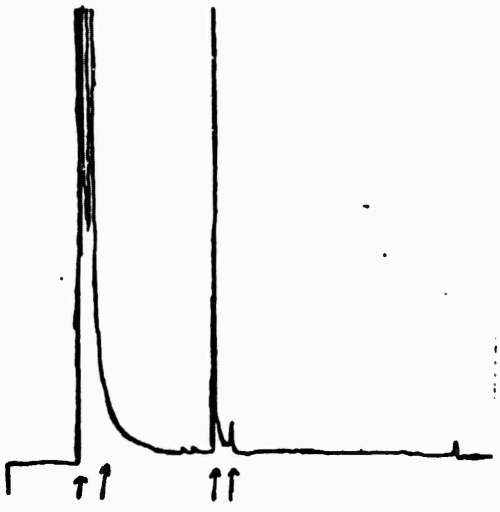
DISCUSSION

Levan was initially chosen for study because of its biodegradability, potential film forming capabilities and production from fermentation methods. The acid labile nature of levan may be advantageous for specific applications where on-demand degradation is desireable.



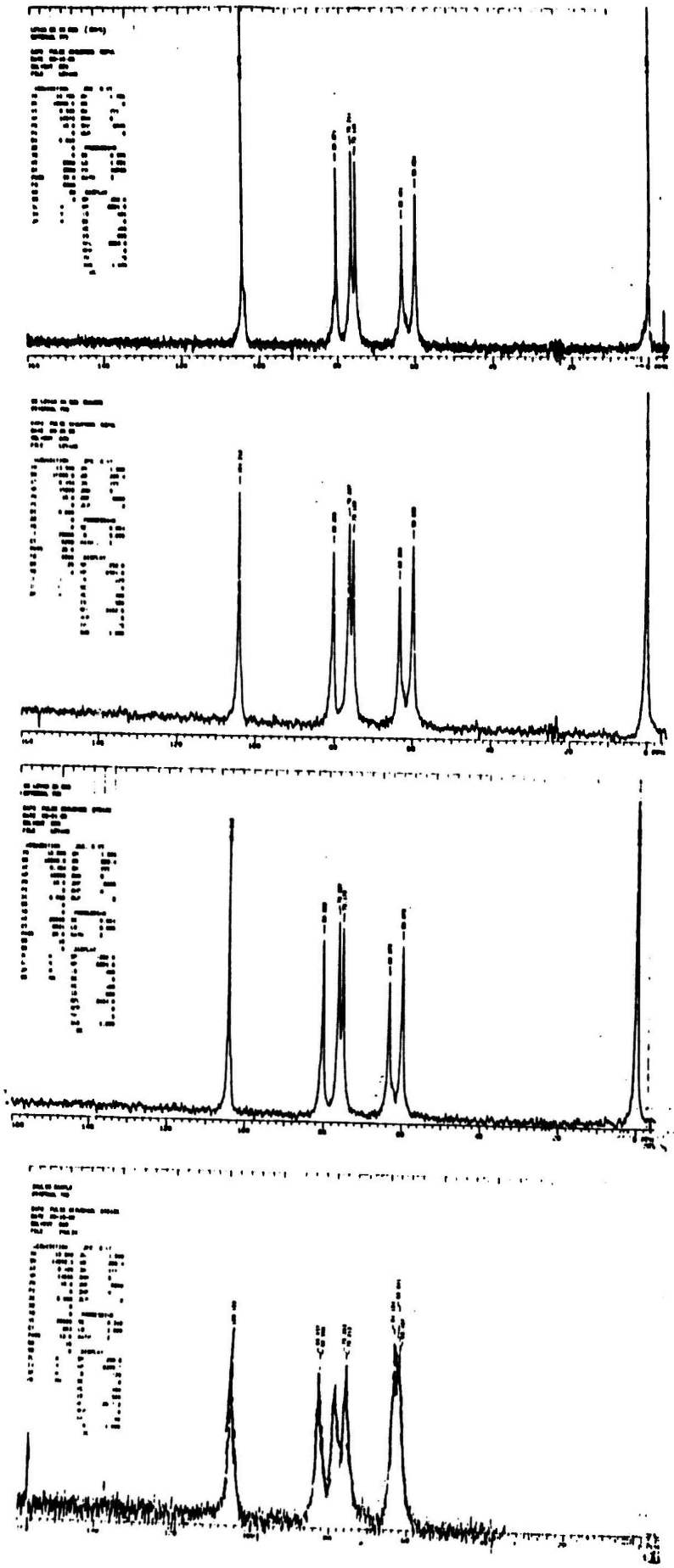
Fructose Standard

4 fructose peaks



Levan Solution

Figure 4. Liquid chromatographic analysis of sugars in the continuous culture system polymer



Levan from Bacillus polymyxa[24]

Levan standard

Continuous culture Tangential Flow purified levan (this study)

Inulin standard

Figure 5. NMR analysis of polymers

Culture Conditions

Studies of the effect of incubation time show that maximum yield of polymer is reached at approximately three days (72 hours) in batch culture. In addition, polymer recovered from cultures incubated longer than three days began to discolor, turning brown. Avigad points out that the production of levanhydrolase could also contribute to a drop in yield over time.[1] In the study reported here only during continuous culture was levan yield maintained over time.

Continuous culture resulted in the highest yield of clean polymer. This may be because the continuous flow system kept the levenhydrolase concentration low and thus avoided the breakdown of already formed polymer. No evidence was found in the literature of attempts to produce levan in continuous culture systems.

Media

Carbon, nitrogen, and phospherous source studies showed that commercial grade sucrose resulted in the greatest yield of the carbon sources evaluated, in agreement with the literature.[4,9,13,19] Urea and a peptone/yeast extract combination resulted in the highest yield of levan for the nitrogen sources evaluated, but the levan produced with urea was whiter (purer) than the product produced with peptone/yeast extract. With the phosphate sources, although 0.2% KH_2PO_4 resulted in the highest yield of levan, 0.3% KH_2PO_4 resulted in the highest yield of clean product. A literature search revealed no previous comparisons of nitrogen and phosphate sources.

Optimum pH for maximizing yield was between 6 and 7. The strain used in this study was slow to adapt to new culture conditions; it took several days to recover, when transferred from a 10% to a 5% sucrose medium. Transfer of this strain from the pH 6.5 inoculum to the pH 5 and 8 culture conditions may have shocked the organism and been at least partially responsible for the poor yields at these more extreme pH values. As pH rose Matoon, et al. found that levan hydrolase activity also increased[5] which could account for the low yields at high pH.

Analysis

The processing method finally developed yielded a clean, pure polymer; however the levan did not solubilize well for GPC analysis in either a sodium acetate buffer system or an alternate Milli-Q water with 0.05% sodium azide system. Since the polymer did not form a true solution, extensive analysis of MW was difficult. Bodie, et al. found that the highly branched structure of levan contributed to its tendency to form gels, inhibiting solubility. In addition, this branched structure makes the properties of levan extremely sensitive to heat and acid hydrolysis.[4]

A sample of the levan isolated from the continuous culture system, for which a small peak was obtained by GPC analysis and the MW was determined at 1507K with a dispersity of 9.0, was also determined by LALLS to have a MW of 125,000K. The sample provided to us by Dr. Han[23,24] also showed the same behavior, giving a GPC weight of 1600K and a LALLS weight of 100,000K. It is difficult to compare these values to other light scattering results from the

literature, as none are reported with any degree of confidence. Mattoon, et al. tried to determine MW by light scattering and found it unreliable because of the agglomeration of the levan upon attempts to redissolve it.[5]

A survey of the literature shows MWs varied with method of analysis. The oldest method, methanol fractionation, gives molecular weights under 200,000 daltons.[5] Gel permeation chromatographic analysis gives values ranging from 1 to 20 million daltons, for levan from Streptococcus salivarius[6] and Erwinia herbicola.[22] Viscosity, light scattering, and sedimentation methods give values for levan Streptococcus salivarius from 18 to 100 million daltons.[20,25,26,27]

Liquid chromatographic analysis of the component sugars confirmed that the polymer was a fructosan, while NMR analysis identified it as levan.

Summary

The optimized conditions of pH, incubation time, culture media, and polymer purification were determined for the production of levan by Erwinia herbicola. While continuous culture fermentation showed promise, MW determinations on the isolated polymer could not be made with certainty. In addition, the yields of polymer were low (actual yields were <10% based on sucrose and gross yields never exceeded 27%) when compared to other biopolymers studied. The physical properties of clear films produced from this polymer could not be adequately evaluated due to brittleness, which was unimproved by plasticizing with glycerol.

CONCLUSIONS

Fermentation and processing conditions for the production of the biopolymer levan by Erwinia herbicola were determined. The polymer can be produced and isolated in a continuous culture fermentation system using Levan Media at a pH of 7.2, but the low yield of polymer and the brittleness of films have resulted in the termination of research on this biopolymer.

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